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RESPONSE ACTIVITY OF ALVEOLAR MACROPHAGES IN PULMONARY DYSFUNCTION CAUSED BY *Leptospira* INFECTION

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ABSTRACT: Leptospirosis is a syndrome with different clinical manifestations including the most severe and often fatal forms of pulmonary disease of unknown etiology. Pulmonary injury during the inflammatory process has been associated with the excessive number of alveolar macrophages (AMs) and polymorphonuclear leukocytes stimulated in the lungs and with the production of reactive oxygen and nitrogen intermediates and other inflammatory mediators. The aim of the present work was to evaluate the cellular immune response of AMs or inflammatory cells of hamsters during leptospirosis. The activity of AMs was determined by measuring nitric oxide (NO) and protein production as well as inflammatory cell infiltration in bronchoalveolar lavage (BAL) fluid. Pulmonary activity during infection was monitored by measuring pH, pressure of oxygen (PaO₂), and pressure of carbon dioxide (PaCO₂) in blood samples. Cellular immune response and its role in the genesis of leptospirosis have been incriminated as the main causes of tissue and pulmonary injuries, which consequently lead to the pulmonary dysfunction in severe cases of leptospirosis. The present results show a low production of NO in both supernatant of alveolar macrophage culture and BAL. In the latter, protein production was high and constant, especially during acute infection. Total and differential cell count values were 2.5X10⁶ on day 4; 7.3X10⁶ on day 21; and 2.3X10⁶ on day 28 after infection, with lymphocytes (84.04%) predominating over neutrophils (11.88%) and monocytes (4.07%). Arterial blood gas analysis showed pulmonary compromising along with the infectious process, as observed in parameter values (mean±SD) evidenced in the infected versus control group: PaO₂ (60.47mmHg±8.7 vs. 90.09mmHg±9.18), PaCO₂ (57.01mmHg±7.87 vs. 47.39mmHg±4.5) and pH (7.39±0.03 vs. 6.8±1.3). Results indicated that *Leptospira* infection in hamsters is a good experimental model to study leptospirosis. However, some of the immune parameters showed variations which might be associated with the animal species.

KEY WORDS: alveolar macrophages, bronchoalveolar lavage, gasometry, pulmonary dysfunction, hamsters, *Leptospira*.

CONFLICTS OF INTEREST: There is no conflict.

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INTRODUCTION

Leptospirosis is a zoonotic infection caused by spirochetes of the genus *Leptospira*, which is common in the tropics and in developing countries (1). Animal reservoirs, particularly rats, are the most frequent disseminators of the disease worldwide by excreting spirochetes in the urine. *Leptospira* can enter the body through skin wounds, including very small scratches, as well as through intact mucous membranes of the mouth, nose and eyes (7). The lung is a target of leptospiral infection, which develops hemorrhagic pneumonitis of different severity (6). Pulmonary injury during the inflammatory process has been associated with the excessive number of alveolar macrophages (AMs) and polymorphonuclear leukocytes, which are active in the lungs, and with the production of reactive oxygen and nitrogen intermediates as well as other inflammatory mediators. The AMs constitute one of the first lines of cellular defense against inhaled pathogenic material and have a high phagocytic and microbicidal potential. Increasing evidence indicates that nitric oxide (NO) released by AMs plays a role in significant pulmonary injury, at least in some species (3). Marinho *et al.* (8) observed the role of cytokines, H₂O₂ and NO in the immunopathology of leptospirosis in genetically selected mice and suggested that factors related to the inflammatory response are associated with the immunopathogenesis of the disease. The present study investigated aspects of cellular immune response of both AMs and inflammatory cells in hamsters infected with *Leptospira*.

MATERIALS AND METHODS

Inoculum

The *Leptospira interrogans* serogroup Icterohaemorrhagiae sample (strain n° 11437) was provided by the Oswaldo Cruz Foundation (FIOCRUZ) Laboratory and maintained in semi-solid Fletcher medium.

Animals

This study was approved by the local Animal Care and Use Committee of Federal University of São Paulo, Brazil. Male golden hamsters (*Mesocricetus auratus*), weighed between 120 and 150g, were randomly allocated into equal groups (n=5). Each experimental set was composed of a group of inoculated hamsters (n=5) infected with 10³ *Leptospira* of virulent culture in EMJH medium by intraperitoneal

(i.p.) injection. The negative control group of five inoculated hamsters was injected with 1ml EMJH medium alone.

Animal Blood Collection and Sacrifice

To analyze arterial blood gas tension values, the animals were anesthetized i.p. with Thiopental (Tiopentax). Blood (0.3ml) was sampled by left ventricle puncture using a heparinized syringe, immediately sent in ice to the laboratory and analyzed by blood gasometry (Roche Compact 3®). The animals were immediately sacrificed using a lethal dose of Thiopental (100mg/kg, i.p.) 4, 7, 14, 21 and 28 days after infection.

Bronchoalveolar Lavage (BAL)

The animals were subjected to tracheostomy and a cannula was inserted into their trachea. The thorax was opened, the left lung was tied and the right lung was washed three times with 20ml/kg sterile phosphate-buffered saline solution (PBS). The returned volume was assigned. Lavage fluid was centrifuged at 300Xg for 10 min, and the resulting cell pellet was washed twice and resuspended in RPMI 1640 (Sigma Co) supplemented with L-arginine, gentamycin (0.16mg/ml), and 10% heat-inactivated fetal bovine serum (Sigma Co).

Cell Count

A 100µl aliquot was used for the total cell concentration in the BAL. Stains were determined in a Neubauer chamber under a light microscope at 100X magnification.

Differential cell counts

Counts were performed after cytocentrifugation and May-Grünwald-Giemsa staining using a light microscope at 400X magnification. The preparations contained about 97% to 100%. AMs were characterized by morphologic criteria. The cell viability of AMs was determined by trypan blue exclusion and was greater than 90%.

Cell Isolation and Culture

Lavage cells, 0.2×10^6 cells/well, were plated in 96-well flat bottom cell culture plates (Maxisorp-Nalge Nunc International IL-USA) and incubated for 2h at 37°C in 5% CO₂/95% air. After removing non-adherent cells with RPMI 1640 medium at 37°C, the adherent cells were incubated in 200µl of culture medium.

The AMs were treated with or without 100 μ l of *Salmonella abortus equi* lipopolysaccharide (LPS; 100U/ml) for 24h at 37°C and in 5% CO₂/95% air.

Measurement of Nitrogen Reactive Species

NO content was quantified by measuring its oxidation product nitrite using the Griess reaction by a microplate assay method (5). After i.p. inoculation with 0.5ml of *L. interrogans* serogroup Icterohaemorrhagiae in the hamsters, NO production by AMs was determined by two methods: (a) using BAL fluid; and (b) using the supernatant of AMs culture obtained after 24h incubation. Cell cultures were either stimulated or non-stimulated with *Salmonella abortus equi* (100U/ml). Nitrate in the samples was quantified by interpolation on a standard curve of sodium nitrite at 540nm. All samples ranging from 0 to 200 μ M were analyzed in triplicate.

Protein Determination

Protein concentrations in BAL fluid were determined as previously described (14) by using the bicinchoninic acid (BCA) assay method with bovine serum albumin (Sigma Co) as the standard.

Statistical Analysis

Statistical analysis was carried out using the SPSS statistical software (SPSS 10.0, SPSS Science, Chicago, IL, USA). Data are reported as means \pm SD. Values were compared by ANOVA followed by the Tukey-Kramer, multiple comparisons test with the level of significance set at $p < 0.05$ (17).

RESULTS

Arterial Blood Gas Measurement

The values of PaO₂, PaCO₂ and pH were significantly increased when compared with their respective controls on days 4 (59.53mmHg \pm 4.53), 14 (57.73mmHg \pm 10.51) and 28 (51.20mmHg \pm 12.31). The values of PaO₂ and pH were increased on days 21 (7.38 \pm 0.02) and 28 (7.38 \pm 0.01), respectively. There was no significant difference through 28 days of the study among the infected groups. PaO₂ was higher in the infected animals than in controls on days 14 (63.40 \pm 6.87) and 28 (67.00 \pm 3.54). pH was increased in the infected groups only on day 7 post-infection (7.45 \pm 0.04).

PaO₂ and PaCO₂ in control groups and in animals infected with *Leptospira interrogans* serogroup Icterohaemorrhagiae are shown in Table 1 and Figures 1 and 2, which illustrate the kinetics of pulmonary compromising over the 28-day infection period.

Total and differential cell counts in BAL fluid

Total cell count was similar between infected and non-infected animals over the entire study period. In control animals, the cell count was of the same magnitude on days 4 and 7 (2.5×10^6), with a small increase observed on day 21 (3×10^6) followed by a decrease on day 28 (2.3×10^6); no count was done on day 14. In infected animals, a lower cell count was obtained at the initial phase (2.5 , 4.0 and 1.5×10^6 on days 4, 7 and 14, respectively) with subsequent increases on days 21 and 28 (12 and 30×10^6 , respectively). The differential cell count in BAL fluid showed the absolute predominance of lymphocytes (84.04%), followed by neutrophils (11.88%).

Nitrogen Reactive Species

Figures 3 and 4 show that the NO production, determined in both BAL fluid and AMs culture supernatants was below the detection limit (0.5nmol) in control and experimental groups, respectively.

Protein Concentration in BAL

Protein concentration in BAL fluid of control and infected hamsters increased significantly from the fourth day after infection until day 21 and decreased by day 28 post-infection (Figure 5). Table 1 and figure 6 show the mean pH (\pm SD) in blood samples.

Table 1. Mean arterial oxygen tension, arterial dioxide carbon tension and arterial pH (mean±SD) in hamsters inoculated with *Leptospira interrogans* Icterohaemorrhagiae and their respective control group.

Variable	Day	Control	Infected
PaO ₂ (mmHg)	4	88.10 A	59.53±4.53 aB
	7	92.60 A	74.95±14.50 aA
	14	90.50 A	57.73±10.51 aB
	21	76.85 A	58.97±9.27 aA
	28	102.40 A	51.20±12.31 aB
PaCO ₂ (mmHg)	4	42.80 A	51.97±4.23 bA
	7	42.40 A	48.35±3.04 bA
	14	49.60 B	63.40±6.87 aA
	21	52.45 A	54.37±4.39 bA
	28	49.70 B	67.00±3.54 aA
PH	4	7.40 A	7.39±0.02 bA
	7	7.46 A	7.45±0.04 aA
	14	7.40 A	7.36±0.02 bA
	21	7.30 B	7.38±0.02 bA
	28	7.45 A	7.38±0.01 bB

Means with different lowercase letters in a column are significantly different (p<0.05).

Means with different uppercase letters in a row are significantly different (p<0.05).

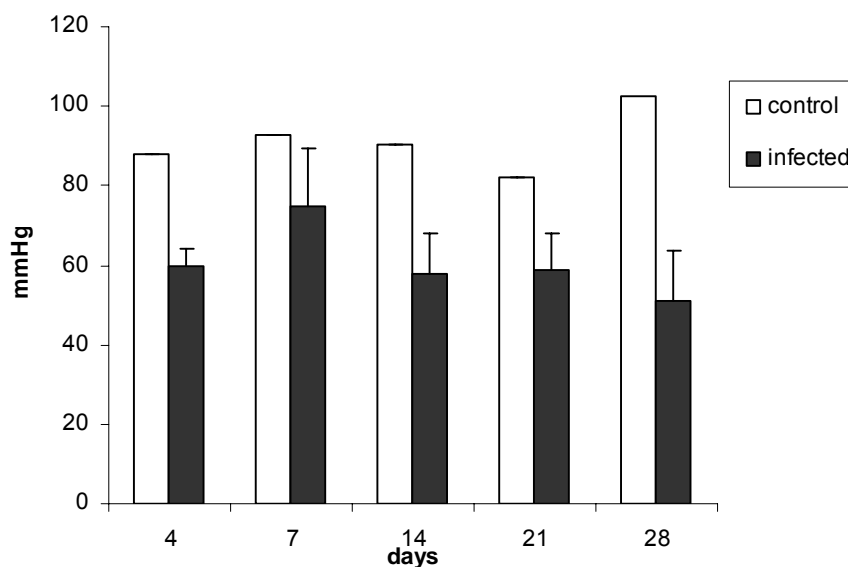


Figure 1. Mean arterial oxygen tension ($PO_2 \pm SD$) in blood samples of hamsters inoculated with *Leptospira interrogans* Icterohaemorrhagiae and their respective control group.

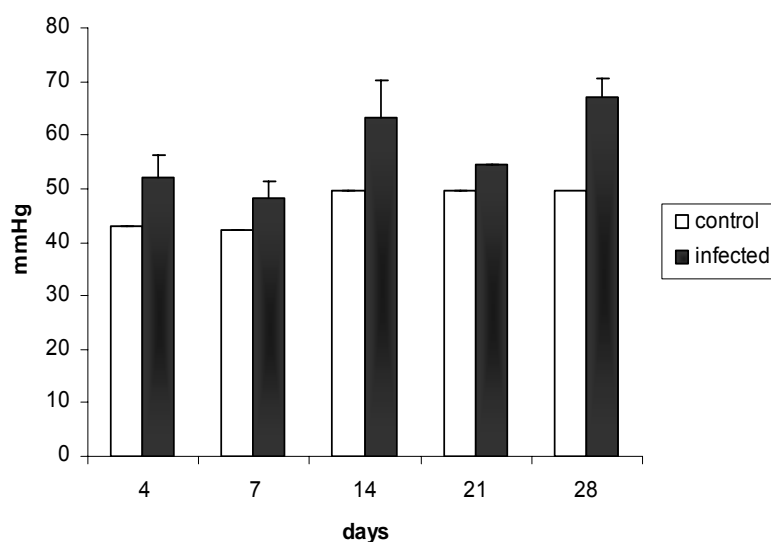


Figure 2. Mean arterial carbonic gas tension ($PCO_2 \pm SD$) in blood samples of hamsters inoculated with *Leptospira interrogans* Icterohaemorrhagiae and their respective control group.

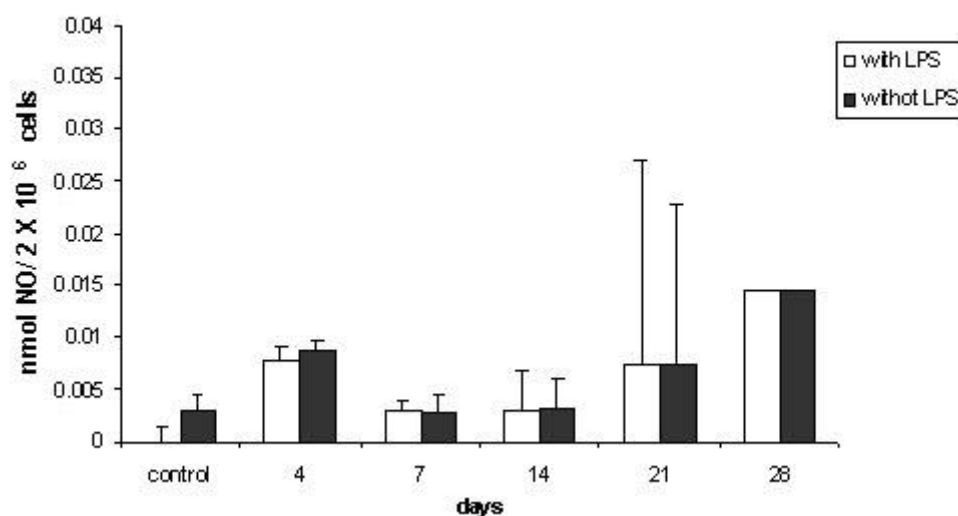


Figure 3. Mean NO concentrations (\pm SD) in the culture supernatant with or without LPS stimulation for 24 hours in hamsters inoculated with *L. interrogans* Icterohaemorrhagiae and their respective control group.

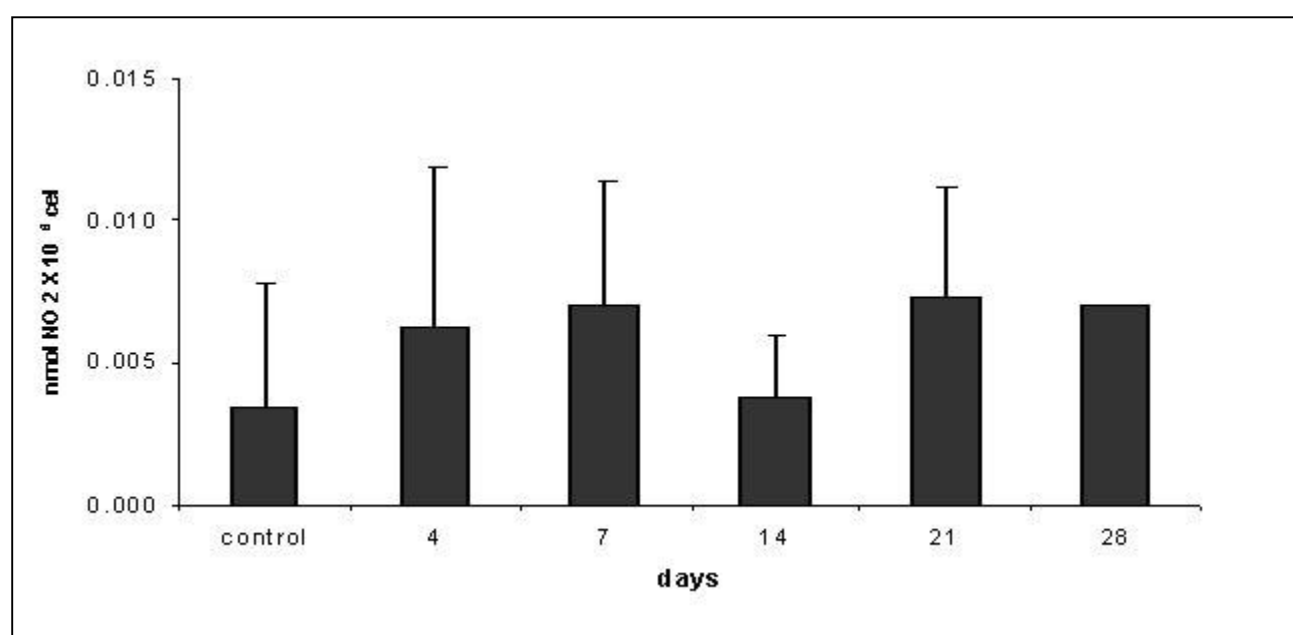


Figure 4. Mean NO concentrations (\pm SD) in the bronchoalveolar lavage fluid of hamsters inoculated with *L. interrogans* Icterohaemorrhagiae and controls.

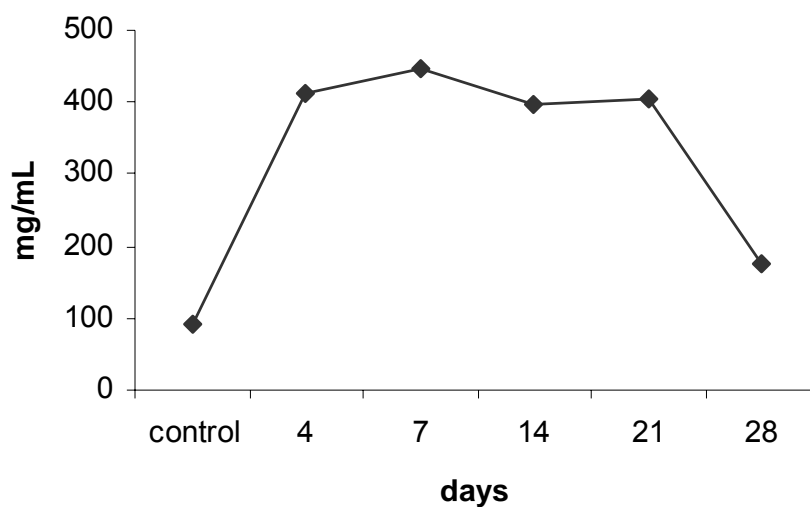


Figure 5. Protein levels present in bronchoalveolar lavage fluid of hamsters infected with *Leptospira interrogans* serogroup Icterohaemorrhagiae and controls.

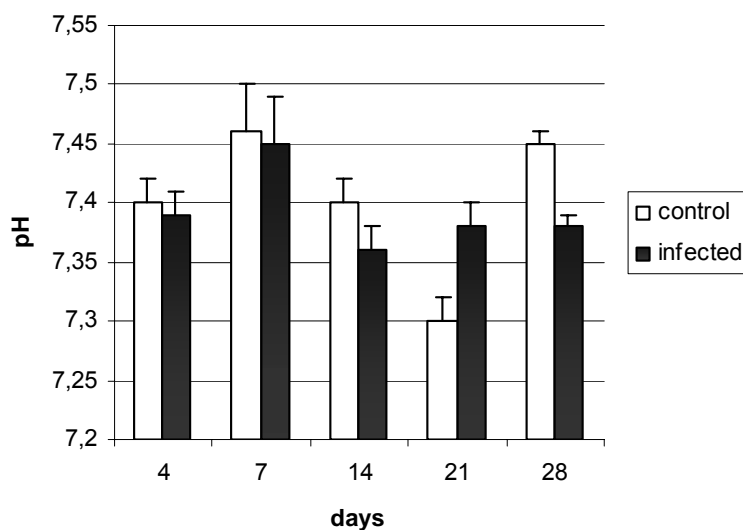


Figure 6. Mean pH (\pm SD) in blood samples from hamsters inoculated with *Leptospira interrogans* Icterohaemorrhagiae and their respective control group.

DISCUSSION

The current study showed that *Leptospira* infection promoted lung dysfunction, as assessed by decreased PaO₂, increased total and differential cell counts after 28 days of infection. *Leptospira* infection enhanced protein production in BAL and NO production by AMs. The latter was considered mild in response to *Leptospira* infection, was similar in cell culture supernatant or in BAL fluid, in the presence or absence of LPS, and had a mild increase when cells were stimulated with LPS or *Leptospira*. Our results suggest that suppression in the cellular immune response by hamster AMs may be associated with an immune incompetence related to this species. According to Dörger *et al.* (4), the inability of phagocytes to produce NO has also been shown for other species, suggesting that leukocyte response to NO production may vary among species, even if they are stimulated with LPS and/or IFN-γ (9, 11). NO production by AMs after stimulation with LPS or other cytokines has been explained in some rodents such as rats and mice (2, 10). *Leptospira* infection in hamsters is considered a good experimental model (15). However, Sakai *et al.* (13) suggested that there is an alteration in PaO₂ and PaCO₂ values, whereas Döger *et al.* (4) reported an alteration in the NO production by AMs. This fact could explain the suppression of the immune response by AMs observed in our study. At high levels, these short-lived molecules are implicated in a variety of tissue injury mechanisms, including: endothelial damage, with thrombosis and increased permeability; protease activation and antiprotease inactivation, with a net increase in breakdown of the extracellular matrix; and direct injury to parenchyma cells such as alveolar cells (9). These injuries may finally lead to hemorrhagic acute respiratory distress syndrome (ARDS) in leptospirosis patients (16). Diffuse pulmonary hemorrhage leading to death is a syndrome which may develop during leptospirosis, but its pathophysiology is not well documented. The relationship between the low NO production and the activation of alveolar macrophages was not clear in the case reported by Yang & Hsu (16), who suggested that an immune-mediated destruction of the alveolar-endothelial tissue may have played a role in the development of severe diffuse pulmonary hemorrhage.

Bronchoalveolar lavage (BAL) is used as a routine diagnostic procedure in pulmonology and is considered a safe and useful method for the assessment of cellular and chemical profiles of the airways that play a role in the immunopathogenesis of inflammatory diseases (2, 12).

The analysis of BAL content reveals which cells and substances become active and are therefore released during the infectious process. Protein content in BAL fluid was increased during most of the experimental periods and decreased drastically at the final phase of the infection. This result indicates that the kinetics of anti-inflammatory response by AMs was compromised.

Differential cell counts in BAL fluid indicated that the hamsters responded to the infectious process, with lymphocytes predominating over neutrophils and monocytes. This is possibly associated with the species used, since Mitruka *et al.* (10) determined a predominance of lymphocytes (54.7–92.3) followed by neutrophils, monocytes and eosinophils (17–57, 0.9–4.0, 0.26–1.54, respectively) in adult male hamsters. In the present study, the large lymphocyte number in BAL fluid is possibly related to an antigenic mimicry or to the presence of a *Leptospira interrogans* glycoprotein.

The results obtained by the serial blood gas measurements showed that, although O₂ levels were low, the control groups differed significantly, which suggests a physiological compromising during gas exchanges. However, hamsters in the normal condition at the baseline had relatively lower PaO₂ (58.7±5.5mmHg) and higher PaCO₂ (55.7±3.8mmHg) than the other animals due to alveolar hypoventilation, a result from their adaptation to a fossorial environment (13).

The results presented here indicate that the hamster AMs lack the ability to express NO in response to *Salmonella abortus equi* LPS or *Leptospira in vitro* as identically reported by Dörger *et al.* (4). Our results also indicate that *Leptospira* infection in hamsters is a good experimental model to study this disease, leptospirosis. However, some of the immune parameters showed variations that might be associated with the animal species. It is well known that in the immunopathogenesis of leptospirosis there is a mobilization of the immune response although mobilization of alveolar macrophages could not be observed in the present study.

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